WESTERN BLOT PROTOCOL

Goal:

Confirm that the protein of interest is expressed in the cell.

SOLUTIONS

Lysis Buffer (for whole bacteria lysis)

65mM Tris pH 6.8 (7.4)->pH depends on the stacking gel
2% SDS
10% Glycerol

Loading Buffer

65mM Tris-HCl pH 6.8 (7.4)-> pH depends on the stacking gel
2% SDS
10% Glycerol
5% mercaptoethanol (add under the HOOD)

1X Tris-Glycine Running Buffer

25mM Tris
190mM glycine
0.1% SDS
Adjust to pH 8.3 (if necessary or 7.4 depending on the gel)

1X Transfer Buffer

25mM Tris
190mM glycine
20% Methanol
Adjust to pH 8.3 (if necessary); if protein is >80kDa then add 0.1% of SDS

Porceau Staining Buffer

0.2% (w/v) Porceau S
5% glacial acetic acid

1X TBST WashingBuffer

20mM Tris
150mM NaCl
0.1% Tween-20

Blocking Buffer Milk

(5%) in TBST OR

BSA (3%) in TBST (if you will use a phospho-Antibody detection)

Antibody Solution

Antibody is diluted in Blocking Buffer (e.g. 1:5000)

Stripping Buffer
20mL 10% SDS
12.5 mL 0.5M Tris-HCl
67.5mL MilliQ water
0.8mL β-mercaptoethanol

PROCEDURE

1. Whole Cell Lysis

- Grow bacteria to and OD600 about 0.5. Take 5 mL of the bacterial solution and centrifuge at 16,000 rpm for 5 min
- Remove the supernatant carefully
- Resuspend the pellet in 50 µL of cold *Lysis Buffer*
- Boil the sample for 10 min at 95°C and freeze them at -20°C for 10min. Repeat this 3 times.

Note: You can aliquot the sample and store it at -20°C or -80°C for long-term storage

2. Protein Separation by Gel electrophoresis

- Add to 10 µL of the sample to 10 µL of Loading Buffer. Add to this 1 µL of bromophenol blue.
- Boil the samples at 95°C for 5 min.
- Quick-spin the sample down.
- Set-up the SDS-PAGE chamber by putting the pre-cast gel in the construct and then fills the tank with *Running Buffer.* Remove the comb in the tank.
- Load equal amounts (20 µL) into the wells of a mini pre-cast gel (Biorad)
- Run the gel at 200V (max. mA) and let the blue marker run down shortly before it reaches the bottom (in the last third of the gel)- Approx 35-45min. Stop the run and get the gel out of it cast. (CAREFUL!!! Gel is very fragile! Don't break it!)
- Cut one corner to remember in which well you have loaded which sample.

3. Transferring proteins from the gel to the membrane

- Let the gel stand in the Transfer buffer for 15 min.
- Prepare the transfer sandwich that consists of 2x sponge, then filter paper, then gel, then nitrocellulose membrane (also cut), then filter paper, then 2x sponges. All steps are done IN A TRANSFER BUFFER BATH.*Note:* Make clear to press out all bubles and close the transfer cassette
- Put the transfer cassette in the transfer tank. Verify that the blot is on the

cathode and the membrane directed to the anode.
- Place also a block in the tank.
- Transfer the proteins at 100V for 30min (this step can immensely influence the bands that you will see later). Alternatively you transfer at 10mA during overnight in the cold room.
-
-
-
-

4. Antibody incubations

- Briefly rinse the blot in water and stain it with Ponceau solution to check the transfer quality.
- Rinse off the Ponceau S stain with three washes with TBST.
- Block in 3% BSA in TBST (required for phopho--Antibodies) or 5% milk in TBST at room temperature for 1 hour.
- Wash three times with TBST.
- Cut the membrane into two pieces: one which will be incubated with the anti- body targeting the target protein; one against a control protein.
- Incubate overnight in the primary antibody solution against the target protein at 4°C.
- *Note:* As control you can use GAPDH-targeting antibody
- *Note:* The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. At room temperature: primery antibody needs about 1-3 hours depending on the antibody quality and performance.
- Recollect the antibody!
- Rinse the blot 3-5 times with TBST for 5 min.
- Incubate the secondary antibody (e.g. HRP-conjugated) for 1 hour at room temperature
- Recollect the antibody!
- Rinse the blot 3-5 times for 5 min with TBST

5. Imaging & Data Analysis

- Apply the chemoluminescent substrate to the blot according to the manufacturers recommendation
- Capture the signal!
- Use the program imagej to read the band intensity of the target protein (in comparison to the loading control GAPDH)

6. Stripping and reprobing
(if the protein sizes are quite different not necessary!!!)

Warm the Stripping Buffer to 50°C

- Add the Stripping Buffer to the membrane in a container designated for stripping. Incubate at 50°C for up to 45min with some agitation.
- Rinse the blot under running water for 1 hour
- Transfer the membrane to a clean container, wash 5 times for 5 min with TBST
- Block in 3% BSA or 5% Milk in TBST at room temperature for 1 hour.
- Incubate with the primary antibody overnight. (Recollect the antibody!)
- Rinse the blot for 3-5 times for 5 min with TBST.
- Incubate with secondary antibody (e.g. HRP-conjugated) for 1 hour at room temperature.
- Rinse the blot 3-5 times for 5 min with TBST.

7. Imaging & Data Analysis

(if the protein sizes are quite different not necessary!!!)

- Apply the chemiluminescent substrate to the blot following the manufacturer's suggestions.
- Capture the chemiluminescent signals
- Use imagej to read the band intensity of the loading control proteins.
- Use the loading proteins levels to normalize the target protein levels.